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(54) Title: RTK/CYTOKINE RECEPTOR CHIMERAS

(57) Abstract

Chimeric receptors consisting of extracellular domains of receptor tyrosine kinases and cytoplasmic domains of cytokines, methods for their production and their use in targeting cytokine responsive cells are described.

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RTK/Cytokine Receptor Chimeras

Neural development appears to be regulated via protein receptor tyrosine kinases (RTK's), which, when activated by their cognate ligand, mediate a phenotypic response such as cell growth, survival or differentiation in such cells. The extracellular portion of each receptor protein tyrosine kinase is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic. Binding of a ligand to the extracellular domain results in signal transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates [Fantl et al. (1992), Cell. **69:** 413-423; Mohammed et al. (1990), Brain Research. **528:** 62-72].

A number of RTK families have been identified based on sequence homologies in their intracellular domain. The receptor and signal transduction pathways utilized by nerve growth factor (NGF) involves the product of the trk proto-oncogene [Kaplan et al. (1991), Nature. **350:** 158-160; Klein et al. (1991), Cell. **66:** 189-197]. Klein et al. [Klein et al. (1989), EMBO Journal. **8:** 3701-3709] reported the isolation of trkB, which encodes a second member of the tyrosine protein kinase family of receptors found to be highly related to the human trk protooncogene. TrkB binds and mediates the functional responses to brain derived neurotrophic factor (BDNF), neurotrophin 4/5 (NT-4/5), and, to a lesser extent, neurotrophin-3 (NT-3) [Ip et al.

(1992), Proc Natl Acad Sci USA. **89:** 3060-3064; Klein et al. (1992), Neuron. **8:** 947-956; Squinto et al. (1991), Cell. **65:** 1-20]. At the amino acid level, the products of trk and trkB were found to share 57 percent homology in their extracellular regions, including 9 of the 11 cysteines present in trk. This homology was found to increase to 88 percent within their respective tyrosine kinase catalytic domains. The Trk gene family has now been expanded to include the trkC locus, with NT-3 having been identified as the preferred ligand for trkC [Lamballe et al. (1991), Cell. **66:** 967-979]

Other RTK families identified to date include the EGF, FGF, Eph, Tyro-3, Ror, PDGF, MET/sea/ron, IgF, Nep/Tyro10, and Ret family. All known growth factor RTKs appear to undergo dimerization following ligand binding ([Schlessinger and Ullrich. (1992), Neuron. **9:** 383-391; Ullrich and Schlessinger. (1990), Cell. **61:** 203-211]; molecular interactions between dimerizing cytoplasmic domains lead to activation of kinase function. In some instances, such as the growth factor platelet derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules [Hart et al. (1993), Nature. **361:** 732-736; Heldin. (1992), EMBO J. **11:** 4251-4259.] while, for example, in the case of EGF, the ligand is a monomer [Weber. (1984), J Biol Chem. **259:** 14631-14636].

The tissue distribution of a particular tyrosine kinase receptor within higher organisms provides relevant data as to the biological function of the receptor. The tyrosine kinase receptors for some growth and differentiation factors, such as fibroblast growth factor (FGF) are widely expressed and therefore appear to play some general role in tissue growth and maintenance. For

example, members of the Trk RTK family [Glass and Yancopoulos. (1993), Trends In Cell Biology. **3:** 262-268] of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting of NGF, BDNF, NT-3 and NT-4/5 (known as the neurotrophins) which bind these receptors promote the differentiation of diverse groups of neurons in the brain and periphery [Lindsay. (1993), Neurotrophic Factors. 257-284.].

The cellular environment in which an RTK is expressed may influence the biological response exhibited upon binding of a ligand to the receptor. Thus, for example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast [Glass et al. (1991), Cell. **66:** 405-413]. Thus, it appears that the extracellular domain provides the determining factor as to the ligand specificity, and once signal transduction is initiated the cellular environment will determine the phenotypic outcome of that signal transduction.

The Trks are somewhat unusual with regard to RTK's in that the receptors as well as at least some of their cognate ligands have been identified. Regions of homology in the Trk's as well as other RTK's, in combination with the use of PCR technology, has rapidly enabled the cloning of an abundant number of novel protein tyrosine kinases, wherein the cognate ligand has yet to be discovered (hence, such receptors are termed "orphan" receptors). For such orphan receptors, despite the lack of cognate ligands, knowledge of the tissues in which such receptors are expressed provides insight into the regulation of the growth, proliferation and

regeneration of cells in target tissues. Because RTKs appear to mediate a number of important functions associated with development and maintenance, their cognate ligands will inevitably play a crucial role in these functions.

The cytokines, including erythropoietin, as well as many of the interleukins, and growth hormone, utilize receptors that, at least initially, have an entirely different intracellular signalling pathway. CNTF, IL-6, LIF, OSM, and IL-11 form a subfamily of cytokines (referred to herein as the "CNTF family") that mediate their biological actions through activation of the related signal transducing receptor β components gp130 and LIFRβ [Stahl and Yancopoulos. (1993), Cell. **74:** 587-590]. Activation of the receptor requires either homodimerization of gp130 for IL-6, or heterodimerization of gp130 with LIFRβ for CNTF, LIF, and OSM [Davis et al. (1993), Science. **260:** 1805-1808; Murakami et al. (1993), Science. **260:** 1808-1810]. Activation of cells that express gp130 and LIFRβ occurs upon exposure to LIF, whereas CNTF also requires interaction with an α–receptor component (which may be in soluble form) to activate gp130/LIFRβ expressing cells. Similarly, IL-6 activates gp130-expressing cells only in the presence of its α-receptor component.

The CNTF family receptor β components preassociate [Stahl et al. (1994), Science. **263:** 92-95] with intracellular tyrosine kinases of the Jak/Tyk family (hereon referred to as the Jaks), which then become activated upon β component dimerization [Lutticken. (1994), Science. **263:** 89-92; Stahl et al. (1994), Science. **263:** 92-95].

Other members of the cytokine receptor superfamily also bind to and activate specific Jaks: receptor components for erythropoietin , growth hormone , GM-CSF, GCSF, and IL-3 use Jak2 [Argetsinger et al. (1993), Cell. **74:** 237-244; Silvennoinen et al. (1993), Proc Natl Acad Sci USA. **90:** 8429-8433; Witthuhn et al. (1993), Cell. **74:** 227-236], IFN-a requires Jak1 and Tyk2 [Muller et al. (1993), Nature. **366:** 129-135; Velazquez et al. (1992), Cell. **70:** 313-322], IFN-g requires Jak1 and Jak2 [Watling et al. (1993), Nature. **366:** 166-170], while IL2 and IL4 use Jak3 [Johnston et al. (1994), Nature. **370:** 151-153; Witthuhn et al. (1994), Nature. **370:** 153-157]. Both gp130 and LIFRβ appear to be unique in that they can utilize distinct combinations of Jak1, Jak2, or Tyk2 in a cell-specific manner [Stahl et al. (1994), Science. **263:** 92-95].

Although the cytokines play a vital role in the hemopoiesis, inflammation and immunity as well as response to infection, their pleiotropic character results in their cross reactivity on many types of cells. Depending upon the cell types with which they react, their potential benefits are often outweighed by their lack of specificity, thus limiting their therapeutic usefulness due to undesirable side effects. For example, LIF is a pleiotropic cytokine that is toxic when administered to animals [Metcalf and Gerating. (1989), Proc Natl Acad Sci. **86:** 5948-5952]. However, a subset of LIF's activities are highly desirable and would be therapeutically worthwhile if they could be specifically activated in a particular cell type.

Accordingly, it would be useful to develop molecules that are able to mimic the effects of specific cytokines on targeted cell

populations, while limiting the cross-reactivity of such cytokines with non-targeted cell populations.

One useful mechanism for accomplishing such targeting, as described herein by applicants, is the use of chimeric receptors whereby the extracellular domain is one utilized by a growth factor, such as a neurotrophin, that recognizes a narrow range of cell populations, and the cytoplasmic domain is one which is capable of initiating signal transduction via a cytokine intracellular signal transduction pathway.

SUMMARY OF THE INVENTION

An object of the invention is to target specific cell populations using chimeric receptors.

Another object of the invention is to stimulate the survival, growth, differentiation and/or proliferation of cytokine responsive cell populations using non-cytokine related growth factors.

An object of the present invention is to provide chimeric receptors consisting of extracellular domains of receptor tyrosine kinases and intracellular domain of the CNTF cytokine family receptor components gp130 and LIFRβ.

Another object of the invention is to provide assay systems for agonists and antagonists of ctyokine related signal transduction pathways.

These and other object are achieved according to the present invention, which is described in greater detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Phosphorylation of STAT3 and PTP1D upon activation of truncated chimeric receptors containing the cytoplasmic domains of gp130 and LIFRβ. A series of truncated chimeric receptors were constructed containing extracellular and transmembrane portions of TrkC fused to the cytoplasmic domains of gp130 (called TG, A) or the extracellular portion of the human EGF receptor fused to the transmembrane and cytoplasmic domains of LIFRβ (called EL, B); each receptor also contained a C-terminal repeat of three 10 amino acid sequence from c-myc that is recognized by the monoclonal antibody 9E10 (indicated by triangle in schematic [Evan et al. (1985), Mol Cell Biol. **5:** 3610-3616]. Vectors encoding these constructs were transfected into COS 7 cells [Stahl et al. (1994), Science. **263:** 92-95]. After 48-72 hours, the COS cells were starved in serum-free medium for 1-2 hours, then stimulated with either NT-3 (50 ng/ml, A and C) or EGF (100 ng/ml, B) for 10 mins, then washed and lysed as previously described [Stahl et al. (1994), Science. **263:** 92-95]. The lysates were sequentially incubated with first 9E10 followed by protein G-sepharose, then anti-phosphotyrosine antibody 4G10 conjugated to agarose (UBI). Proteins immunoprecipitated (IP) with 9E10 were first immunoblotted with anti-phosphotyrosine antibody 4G10; the blots were

subsequently stripped [Boulton et al. (1994), J Biol Chem. **269:** 11648-11655] and reblotted with the anti-myc antibody 9E10. Proteins immunoprecipitated with anti-phosphotyrosine were first immunoblotted with anti-STAT3 [Zhong et al. (1994), Science. **264:** 95-98]; the blots were then stripped and probed with anti-PTP1D (Signal Transduction Labs). All immunoblots were visualized with enhanced chemiluminescence [Boulton et al. (1994), J Biol Chem. **269:** 11648-11655].

Fig. 2. Modular tyrosine motifs added to cytokine receptor cytoplasmic domains can specify STAT3 phosphorylation. (A) A series of truncated receptor molecules were constructed (TGt series) each containing a single tyrosine phosphorylation site in which the gp130 Y1 motif was replaced with each of the indicated tyrosine motifs derived from elsewhere on gp130. (B) Chimeric receptors with TrkC extracellular and transmembrane domain fused to the cytoplasmic domain of murine EPOR were constructed that contain the triple myc epitope tag. Two versions also have sequences encoding 5 additional amino acids as indicated in the schematic inserted between the end of the EPOR sequences, but 5' of the triple myc tag. For both A and B, vectors encoding these constructs were transfected into COS 7 cells and analyzed as described in Fig. 1.

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, chimeric receptors are created containing extracellular and transmembrane portions of a receptor tyrosine kinase (RTK) and the cytoplasmic domains of gp130 or LIFRβ, or, in the alternative, extracellular portions of an RTK, and the transmembrane and cytoplasmic domains of gp130 or LIFRβ.

As used herein, receptor tyrosine kinase refers to any member of the superfamily of transmembrane tyrosine kinases that serve as receptors for a variety of protein factors that promote cellular proliferation, differentiation and survival. Receptor tyrosine kinases include, but are not limited to, the Trks, which bind members of the neurotrophin family, including Nerve Growth Factor (NGF), which preferentially binds TrkA, as well as Brain Derived Neurotrophic Factor (BDNF) and Neurotrophin 4/5, which preferentially bind TrkB, and Neurotrophin-3, which preferentially binds TrkC, as well as the EGF, FGF, Eph, Tyro-3, Ror, PDGF, MET/sea/ron, IgF, Nep/Tyro10, and Ret family..

As used herein, "cytoplasmic domain" refers to the full length cytoplasmic domain of gp130 or LIFRβ, or truncated versions that lack distal portions of the cytoplasmic domain, yet retain the membrane proximal Jak binding domain. For example, as shown in Figure 1, both gp130 and LIFRβ contain 5 tyrosine motifs. Analysis of TrkC:gp130 chimeras has indicated that NT-3, which binds the TrkC receptor, induces tyrosine phosphorylation of two downstream targets of the CNTF family of cytokines-the transcriptional activator STAT3 [Akira et al. (1994), Cell. 77:

63-71; Stahl, et al. (1995), Science, **276:** 1359-1353; Velazquez et al. (1992), Cell. **70:** 313-322] and the tyrosine phosphatase PTP1D [Boulton et al. (1994), J Biol Chem. **269:** 11648-11655].

According to the invention, chimeric receptors made according to the invention are used to enable the RTK dependent survival, growth, proliferation and/or differentiation of target cells, the growth, survival, proliferation and/or differentiation of which are normally influenced or controlled by cytokines.

The widespread expression of gp130 and (to a lesser extent LIFRβ) suggests the widespread existence of cells capable of responding to an RTK cognate ligand when expressing an RTK/gp130 or RTK/LIFRβ chimeric receptor. A target cell for expression of an RTK/gp130 or LIFRβ chimera may be any cell which demonstrates a phenotypic response (such as differentiation, the expression of immediate early genes or the phosphorylation of CLIP or JAK proteins) in response to treatment with the CNTF/receptor complex, the IL-6/receptor complex, LIF or OSM, or a hybrid or mutant thereof that either mimics or alters the normal physiological effect of the CNTF/receptor complex. Examples of cell types which respond to these cytokines include myeloid leukemia cells such the M1 cell line, other leukemia cells, hematopoietic stem cells, megakaryocytes and their progenitors, DA1 cells, osteoclasts, osteoblasts, hepatocytes, adipocytes, kidney epithelial cells, stem cells, renal mesangial cells, T cells, B cells, etc.

The present invention includes the nucleotide sequence that encodes the chimeric receptors described herein, as well as host cells and microorganisms and vectors that carry the

recombinant nucleic acid molecules. Cells that express receptor protein may be genetically engineered to produce receptor, as described supra, by transfection, transduction, electroporation, microinjection, via a transgenic animal, etc. of nucleic acid encoding chimeric receptor in a suitable expression vector. In particular embodiments, the host cell carrying the recombinant nucleic acid is an animal cell, such as COS. In other embodiments, the host cell is a bacterium, preferably Escherichia coli.

Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric receptors described herein. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence encoding the receptor protein or peptide fragment may be regulated by a second nucleic acid sequence so that the receptor protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of receptor may be controlled by any promoter/enhancer element known in the art. Promoters which can be used to control receptor expression include, but are not limited to the long terminal repeat as described in Squinto et al., (1991, Cell **65**:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature **290**:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell **22**:787-797), the herpes thymidine kinase promoter (Wagner et

al., 1981, Proc. Natl. Acad. Sci. U.S.A. **78**:144-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature **296**:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. **75**:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. **80**:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, **242**:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell **38**:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. **50**:399-409; MacDonald, 1987, Hepatology **7**:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature **315**:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell **38**:647-658; Adames et al., 1985, Nature **318**:533-538; Alexander et al., 1987, Mol. Cell. Biol. **7**:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell **45**:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. **1**:268-276), alpha-feroprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. **5**:1639-1648; Hammer et al., 1987, Science **235**:53-58); alpha 1-antitrypsin gene control

region which is active in the liver (Kelsey et al, 1987, Genes and Devel. **1**:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature **315**:338-340; Kollias et al., 1986, Cell **46**:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell **48**:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature **314**:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science **234**:1372-1378).

Expression vectors containing receptor-encoding gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the receptor-encoding gene is inserted within the marker gene sequence of the vector, recombinants containing the gene insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the

foreign gene product expressed by the recombinant vector. Such assays can be based, for example, on the physical or functional properties of the receptor-encoding gene product, for example, by binding of the receptor to neurotrophic factor or to an antibody which directly recognizes the receptor. Cells of the present invention may transiently or, preferably, constitutively and permanently express receptors or portions thereof.

In preferred embodiments, the present invention provides for cells that express receptors described herein or portions thereof and that also contain recombinant nucleic acid comprising an immediate early gene promoter [e.g. the fos or jun promoters (Gilman et al., 1986, Mol. Cell. Biol. **6**:4305-4316)]. When such a cell is exposed to a ligand that binds to the receptor, the binding secondarily induces transcription off the immediate early promoter. Such a cell may be used to detect receptor/ligand binding by measuring the transcriptional activity of the immediate early gene promoter, for example, by nuclear run-off analysis, Northern blot analysis, or by measuring levels of a gene controlled by the promoter. The immediate early promoter may be used to control the expression of fos or jun or any detectable gene product, including, but not limited to, any of the known reporter genes, such as a gene that confers hygromycin resistance (Murphy and Efstratiadis, 1987, Proc. Natl. Acad. Sci. U.S.A. **84**:8277-8281) chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (neo), beta-galactosidase beta-glucuronidase, beta-galactosidase, etc. of detecting or measuring neurotrophin activity.

According to the invention, cells engineered to express a chimeric protein described herein are treated using the ligand specific for the extracellular domain of the chimeric receptor. In a preferred embodiment, cells expressing chimeras utilizing extracellular domains of "orphan receptors" (receptors for which no known cognate ligand has been identified) and cytokine cytoplasmic domains. Such cells are utilized in assay systems to identify cognate ligands of the orphan receptors by measuring a predetermined phenotypic change in the chimeric receptor-expressing cells in the presence of a potential ligand.

In another embodiment of the invention, the chimeric receptors made as described herein are used in assay systems that may be used to identify agents that act as agonists or antagonists of CNTF or other cytokine members of the CNTF receptor family that utilize signal transduction pathways in common with the CNTF receptor family. Cells that express TRK/gp130 or TRK/LIFRβ receptor,
or mutants thereof lacking specific tyrosine motifs, may be exposed to a test agent and the tyrosine phosphorylation of either the β-component(s) or the signal transduction component(s) are compared to the tyrosine phosphorylation of the same component(s) in the absence of the test agent. Such assay systems are useful to identify small molecules that are capable of initiating or blocking signal transduction induced by specific cytokines.

In another embodiment of the invention, cells expressing the β-component of a specific receptor and one or more signal transducing components, may exhibit tyrosine phosphorylation of

the β-component in the absence of any transduction inducing ligand. Such cells are useful to determine the ability of a test agent to inhibit this step in the signal transduction pathway by contacting the cells with test agents and measuring the effect of the test agents on the tyrosine phosphorylation. In one embodiment of the invention, the cytokine domain is "engineered" to contain altered or additional motifs that endow novel activity to the receptor, thereby enabling modulation of downstream signaling pathways activated by the receptor. For example, as described in Example 2, applicants have demonstrated that a short tyrosine motif added to the cytoplasmic domain of the EPO receptor can enable that receptor to mediate tyrosine phosphorylation of STAT3. This motif alone (as expressed by a cell or in a cell-free system) may be useful for selecting agonists or antagonists of cytokines, such as the CNTF family, that specifically interact with the STAT3 related segment of the signal transduction pathway.

In another embodiment, chimeric receptor expressing cells engineered in vivo or engineered in vitro or ex vivo may be implanted and subsequently treated with a ligand recognizing the extracellular domain. Ligands capable of recognizing chimeric receptors expressed by a targeted cell population may then be used to treat diseases or disorders which involve the targeted cells, or alternatively, to enhance the growth and/or survival or other function of targeted cells.

The chimeric receptors may be supplied to the system using appropriate vectors, or they may be produced within the system via, for example, appropriately engineered precursor cells. The presence of a chimeric receptor responsive to, for example, a neurotrophin, would enable use of the neurotrophin to elicit a response in cell or tissue cultures, in whole animals, in particular cells or tissues within whole animals or tissue culture systems, or over specified time intervals (including during embryogenesis) in embodiments in which the chimera expression is controlled by an inducible or developmentally regulated promoter. In particular embodiments of the invention, the CMV promoter may be used to control expression of a TRK/cytokine chimeric receptor in transgenic animals. The term "transgenic animals," as used herein, refers to non-human transgenic animals, including transgenic mosaics, which carry a transgene in some or all of their cells, which include any non-human species, and which are produced by any method known in the art, including, but not limited to microinjection, cell fusion, transfection, electroporation, etc. For example, the animals may be produced by a microinjection of zygotes by a method such as that set forth in "Brinster et al. [1989, Proc. Natl. Acad. Sci. U.S.A. **82**:4438-4442]. Techniques for gene therapy or implantation of genetically engineered cells are known in the art. For example, U.S. Patent No. 5,328,470 issued July 12, 1994 entitled "Treatment of diseases by site-specific instillation of cells or site-specific transformation of cells and kits therefore" describes utilization of a catheter type apparatus for

administration of vectors encoding specific genes as well as site-specific placement of engineered cells.

In a preferred embodiment, RTK:gp130 or RTK:LIFRβ chimeras are expressed by cells such that neurotrophins may be utilized in place of LIF to activate the cells. This is of particular use in view of the fact that although LIF has desirable therapeutic properties, it is toxic when administered to animals. [Metcalf and Gearing. (1989), Proc Natl Acad Sci. **86:** 5948-5952]. The use of the chimeric RTK:gp130 or RTK:LIFRβ is a novel way to specifically activate the LIF/IL-6 signal transduction pathway in a certain subset of all LIF responsive cells. For example, LIF has a profound effect on bone morphology, resulting in an increase in both the number of osteoblastic cells as well as an increase in the amount of bone deposition [Evans et al. (1994), Biochem Biohpys Res Commun. **199:** 220-226; Metcalf and Gerating. (1989), Proc Natl Acad Sci. **86:** 5948-5952; Rodan et al. (1990), Endocrinology. **127:** 1602-1608]. Thus individuals with osteoporosis who suffer from bone loss would benefit from the specific stimulation of the osteoblastic cell lineage by LIF. The effect of LIF on osteoblastic cells appears to be direct as osteoblastic cell lines bind and respond to LIF in vitro [Evans et al. (1994), Biochem Biohpys Res Commun. **199:** 220-226; Rodan et al. (1990), Endocrinology. **127:** 1602-1608]. Thus, specific expression of a chimeric RTK:cytokine receptor such as TrkC:gp130 in the osteoblastic cell lineage would enable control of the level of osteoblastic activity and thus the rate of bone deposition through controlling the NT3 levels in the cells environment. NT-3 would be desirable in this regard as the

stimulating factor since there are few natural NT-3 targets outside of the nervous system; thus the chimeric TrkC:gp130 receptors would be a rather specific target of NT-3 in the periphery.

According to the invention, transgenic mice in which the TrkC:gp130 cDNA is controlled by a promoter that is only expressed in cells of the osteoblastic lineage may be created. One such promoter is that controlling osteocalcin, which is only expressed by mature osteoblasts . Transgenic mice in which a foreign gene is driven from the osteocalcin promoter have been derived previously, and they have been shown to express the transgene with the same specificity as the endogenous osteocalcin [Baker et al. (1992), Mol Cell Biol. **12:** 5541-5547; Kesterson et al. (1993), Mol Endocrinol. **7:** 462-467]. Ovariectomized mice develop osteoporosis [Jilka et al. (1992), Science. **257:** 88-91]; accordingly, this model system is used to demonstrate the applicability of treatments of osteoporosis in post menopausal women. Use of NT-3 on TrkC:gp130 transgenic animals can demonstrate the effectiveness of controlling the overall balance of bone formation and loss in an animal model of a human disease.

In another embodiment of the invention, the cDNA encoding the chimeric receptor may be introduced via a precursor cell from which osteoblastic cells arise. A precursor cell that is capable of differentiating into an osteoblastic cell in culture has recently been purified from mice by fluorescence activated cell sorting (FACS) [Van Vlasselaer et al. (1994), Blood. **84:** 753-763]. Osteoprogenitor cell cultures may be transfected in vitro with

DNA encoding a chimeric RTK:cytokine receptor such as TrkC:gp130. The cDNA for TrkC:gp130 is placed downstream of an osteoblastic-specific promoter such as the osteocalcin promoter described above, or using a more general promoter that would be expressed in all of the cells of the lineage such as that for alkaline phosphatase or β-actin. Having developed a stable osteoprogenitor population that expresses the TrkC:gp130 chimeric receptor, these cells are reintroduced into bone marrow. Systemic administration of NT-3 would then be used to mimic the effect of LIF and induce net bone deposition.

In another embodiment of the invention, chimeric receptors as described herein are introduced into, for example, stem cells, simultaneously with the introduction of genes used for correction of human diseases via gene therapy. Thus, for example, hematopoietic stem cells engineered to express human adenosine deaminase (ADA) [Luskey, B., et al. (1990) Ann. N.Y.Acad.Sci. **612**:398-406] may be simultaneously engineered to express chimeric receptors according to the present invention, thereby enabling use of, for example, neurotrophins, to support the transplanted stem cells in vivo.

EXAMPLES

Most cells express endogenous gp130 and LIFRβ, therefore we constructed chimeric receptors to facilitate mutational analysis of their cytoplasmic domains. Since activation of gp130 by IL-6 involves homodimerization of gp130 [Davis et al. (1993), Science. **260:** 1805-1808; Murakami et al. (1993), Science. **260:**

1808-1810], we employed extracellular domains from receptor tyrosine kinases such as TrkC and EGF receptor that dimerize upon binding neurotrophin 3 (NT-3) or EGF respectively.

MATERIALS and METHODS

Chimeric receptors were prepared using method known in the art [Ohashi, et al., (1993) Proc. Natl. Acad. Sci. USA **91**:158-162]. The cloning of LIFRβ and gp130 are described in Gearing et al. (1991) EMBO J. **10**:2839-2848 , Hibi, et al. (1990) Cell **63**:1149-1157 and in published PCT application WO 93/10151 published May 27, 1993, all of which are incorporated by reference in their entirety herein. EGF/LIFRβ chimeras were constructed using amino acids 1-646 of EGF [Collins, et al. (1986) J. Cell. Phys. **137**:293-298] and amino acids 834 to the end of the LIFRβ receptor. ELΔY_5 comprises amino acids 1-646 of the EGF receptor and amino acids 834-1027 of LIFRβ. ELΔY_{4-5} comprises amino acids 1-646 of the EGF receptor and amino acids 834-1000 of LIFRβ. ELΔY_{3-5} comprises amino acids 1-646 of the EGF receptor and amino acids 834-980 of LIFRβ. ELΔY_{1-5} comprises amino acids 1-646 of the EGF receptor and amino acids 834-948 of LIFRβ.

TrkC/gp130 chimeras (TG) were constructed by fusing the N-terminal portion of the trkC receptor up to the unique Sal1 restriction site to amino acids 644 to the end of the gp130 receptor protein. TGΔY_{4-5} comprises the same extracellular segment derived from TrkC and amino acids 644 to 904 of gp130. TGΔY_{3-5} comprises the same extracellular segment derived from

TrkC and amino acids 644 to 813 of gp130. TGΔY_{2-5} comprises the same extracellular segment derived from TrkC and amino acids 644 to 765 of gp130. TGΔY_{1-5} comprises the same extracellular segment derived from TrkC and amino acids 644 to 757 of gp130.

EXAMPLE 1

As predicted, fusion proteins (called TG for TrkC:gp130 or EG for EGFR:gp130) containing these extracellular domains linked to the cytoplasmic domain of gp130 expressed in COS cells gave factor-induced tyrosine phosphorylation of the chimeric receptor and the associated Jak (Fig 1A and data not show). Although there is no known cytokine that induces homodimerization of LIFRβ, a fusion protein (EL for EGFR:LIFRb) containing the EGFR extracellular domain and the transmembrane and cytoplasmic domains of LIFRβ gave robust tyrosine phosphorylation of the chimeric receptor and the associated Jak (Fig 2B). This provides further evidence for the conclusion that LIFRβ functions as a full signal transducing partner to gp130 [Stahl and Yancopoulos. (1993), Cell. **74:** 587-590], and indeed raises the possibility that an as yet undiscovered cytokine could induce downstream signaling through homodimerization of LIFRβ.

EXAMPLE 2

In order to investigate the contribution of the cytoplasmic domains of gp130 and LIFRβ in the activation of

downstream signal transduction pathways, we constructed a series of epitope-tagged chimeric receptor truncations that lacked distal portions of the gp130 or LIFRβ cytoplasmic domains (referred to in the figures as Y_1 through Y_5), yet retained the membrane proximal Jak binding domain. The tyrosine motifs for gp130 are as follows: Y_1=QYSTV; Y_2=GYRHQ; Y_3=QYFKQ; Y_4=SYLPQ; and Y_5=GYMPQ. The tyrosine motifs for LIFRβ are as follows: Y_1=SYCPP; Y_2=IYIDV; Y_3=MYQPQ; Y_4=GYKPQ; and Y_5=GYRPQ. Each of these chimeric receptors were then expressed in COS cells, and analyzed for their ability to induce tyrosine phosphorylation of two downstream targets of the CNTF family of cytokines-the transcriptional activator STAT3 [Akira et al. (1994), Cell. **77:** 63-71; Boulton et al. (1994), Submitted. ; Zhong et al. (1994), Science. **264:** 95-98] and the tyrosine phosphatase PTP1D [Boulton et al. (1994), J Biol Chem. **269:** 11648-11655]. STAT3 is preferentially activated by the CNTF cytokine family relative to other cytokines such as IFN-g, GM-CSF, or IL-3, and the receptor sequences that might be required for its activation are unknown [Boulton et al. (1994), Submitted.]. Since both PTP1D and STAT3 contain SH2 domains that could potentially interact with phosphorylated tyrosines on gp130, LIFRβ, or the associated Jak, the receptor truncation sites were chosen to successively remove the 5 distal tyrosines in gp130 or LIFRβ.

Analysis of the TG chimeric receptor truncation series reveals that both STAT3 and PTP1D are inducibly tyrosine phosphorylated (disclaimer) upon stimulation with NT3 regardless of whether the cytoplasmic domain lacks the three distal tyrosine motifs (Fig 1A). However, further truncation to remove the Y2

motif creates a receptor that has lost the ability to mediate STAT3 phosphorylation, yet retains the ability to phosphorylate PTP1D. Further truncation to remove the Y1 motif also eliminates the phosphorylation of PTP1D, despite the fact that the receptor is expressed at equal or greater levels relative to the other truncated receptors, and tyrosine phosphorylation of the associated Jak kinase is still observed. These results implicate the region of gp130 containing the Y2 motif (sequence GYRHQ) in mediating the tyrosine phosphorylation of STAT3, and the Y1 motif (sequence QYSTV) in the tyrosine phosphorylation of PTP1D. The latter sequence is consistent with the consensus tyrosine containing motif proposed for interaction of PTP1D with receptor tyrosine kinases [Case et al. (1994), J Biol Chem. **269:** 10467-10474]. Analysis of chimeric receptors (EL) containing portions of the LIFRβ cytoplasmic domain reveals that dimerization of LIFRβ also results in tyrosine phosphorylation of STAT3 (Fig 1B), but not PTP1D (data not shown). Furthermore, the truncation of the cytoplasmic domain proximal to the Y3 motif results in loss of STAT3 activation, while tyrosine phosphorylation of the chimeric receptor and the associated Jak is still observed (Fig. 1B). These data implicate the region of LIFRβ containing the Y3 motif (sequence MYQPQ) in the tyrosine phosphorylation of STAT3. All together, these results are consistent with the notion that activation of a Jak kinase is insufficient to activate many of the downstream signaling pathways, and that sequences in the cytoplasmic domains of the Jak-binding cytokine receptors are critical for specifying which

downstream substrates are phosphorylated upon receptor activation.

We next constructed point mutations of the TG chimeric receptor in which the Y1 or Y2 tyrosines were changed to phenlyalanine to ascertain whether these residues were essential for induction of tyrosine phosphorylation of PTP1D and STAT3. Subsequent analysis confirmed that the mutant TGY1-F still gave NT3-induced phosphorylation of STAT3, but not PTP1D (Fig. 1C). However, the mutant receptor TGY2-F was still capable of inducing tyrosine phosphorylation of both PTP1D and STAT3 (Fig. 1C). One interpretation of this result is that there are multiple sites in the gp130 cytoplasmic domain that are capable of mediating STAT3 phosphorylation, the last of which is removed in the truncation mutant TGDY2-5. Both of the tyrosine motifs in the cytoplasmic regions of gp130 and LIFRb that were implicated in STAT3 phosphorylation have the sequence YXXQ, a motif which is indeed present in multiple copies in both gp130 and LIFRβ.

To further investigate the requirements for STAT3 activation and examine whether the other tyrosine motifs of gp130 could indeed mediate phosphorylation of STAT3, we constructed a series of epitope-tagged chimeric receptors (the TGt series) in which nucleotide sequences encoding the 7 amino acids encompassing each tyrosine from the Y3, Y4, and Y5 motifs were appended to the TGDY1-5 truncation, which by itself contains no functional tyrosines, does not become tyrosine phosphorylated, and does not give STAT3 activation (Fig 1A). We then transfected these constructs into COS cells, and asked whether any of the truncated receptors containing only these

single tyrosine motifs were capable of mediating STAT3 tyrosine phosphorylation. This analysis showed that both the Y5 and Y4 motifs were capable of mediating STAT3 phosphorylation, while the Y3 motif was not, despite the fact that it was inducibly phosphorylated by NT3 and also gave rise to tyrosine phosphorylation of the associated Jak (Fig 2A). These results indicate that the Y5 and Y4 tyrosine motifs can indeed endow a truncated receptor with the ability to give tyrosine phosphorylation of STAT3. Furthermore, these results further suggest that these short tyrosine motifs are somewhat modular, in that they can be moved from one portion of the gp130 cytoplasmic domain to another, yet still function in mediating STAT3 phosphorylation.

To further explore the modularity and sequence requirements of the tyrosine motifs mediating STAT3 phosphorylation, we constructed another set of epitope-tagged chimeric receptors (TE for TrkC:EPOR) in which the cytoplasmic domain of the erythropoietin receptor was fused to the extracellular domain of TrkC. Furthermore, we also constructed a version in which the 5 amino acids of the gp130 Y5 motif (GYMPQ) were appended to the end of the EPOR cytoplasmic domain. Analysis of these chimeric receptors expressed in COS cells revealed that the cytoplasmic domain of EPOR was incapable of mediating STAT3 tyrosine phosphorylation. However, addition of the 5 amino acid Y5 tyrosine motif from gp130 endowed the EPOR cytoplasmic domain with the ability to mediate tyrosine phosphorylation of STAT3. These results convincingly demonstrate that a short tyrosine motif added to the cytoplasmic

domain of a Jak-binding cytokine receptor component can endow a novel activity to that receptor and can thereby modulate the downstream signaling pathways that each receptor can activate. Furthermore, these short tyrosine motifs are to some extent modular in that they retain function in a variety of locations within the cytoplasmic domain, as well as when embedded within entirely different receptor sequences.

The results presented above suggest that all the potential tyrosine phosphorylation sites examined in gp130 and LIFRb that were capable of mediating STAT3 activation feature a glutamine at the Y+3 position. Indeed, mutation of this residue to alanine eliminates mediation of STAT3 phosphorylation, consistent with a critical role for this residue (Fig 2B). However, there may be further requirements as the gp130 Y3 motif conforms to this general sequence, yet did not mediate STAT3 phosphorylation in the TGtY3 chimeric receptor (Fig 2A). Interestingly, all of the tyrosine motifs that gave STAT3 activation also contained a small amino acid (G, S, or A) at the Y-1 position, with the exception of the LIFRβ Y3, which has methionine at Y-1. Thus sequences that conform to the motif YXXQ could be considered candidates for mediating STAT3 activation, although additional sequences may also be required. Previous analysis of other cytokines showed that GCSF could mediate STAT3 activation, while GMCSF could not [Boulton et al. (1994), Submitted.]. In agreement with the tentative consensus described above, the murine GCSF receptor contains the sequence AYVLQ, while neither the GMCSF receptor nor the EPO receptor have sequences matching this motif.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

WE claim:

1. A chimeric protein receptor comprising:
   a) the extracellular domain of a receptor tyrosine kinase; and
   b) the cytoplasmic domain of gp130 or LIFRβ.

2. A chimeric protein according to claim 1 wherein the extracellular domain (a) is at least one domain selected from TrkA, TrkB and TrkC.

3. A chimeric protein receptor according to claim 1 or 2 wherein the cytoplasmic domain (b) is truncated.

4. A chimeric protein receptor according to claim 3 wherein the truncated cytoplasmic domain (b) comprises one or more tyrosine motifs selected from Y_1, Y_2, Y_3, Y_4 and Y_5 of LIFRβ.

5. A chimeric protein receptor according to claim 3 wherein the truncated cytoplasmic domain (b) comprises one or more tyrosine motifs selected from Y_1, Y_2, Y_3, Y_4 and Y_5 of gp130.

6. An Isolated nucleic acid encoding a protein as defined in any one of claims 1 to 5.

7. A nucleic acid expression vector comprising a nucleic acid according to claim 6.

8. A cell harbouring a nucleic acid according to claim 6 or 7.

9. A cell according to claim 8 which expresses a chimeric receptor to any one of claims 1 to 5.

10. A method of producing a chimeric protein receptor according to any one of claims 1 to 5 comprising culturing a cell according to claim 8 or 9.

11. A chimeric protein receptor produced by the method of claim 10.

12. A chimeric protein receptor according to any one of claims 1 to 5 or 11, or a nucleic acid according to claim 6 or 7, or a cell according to claim 8 or 9; for use in a method of treatment of the human or animal body; or for use in a method of diagnosis.

13. A chimeric protein receptor according to claim 1, 11 or 12 substantially as hereinbefore described with reference to any one of the foregoing Examples.

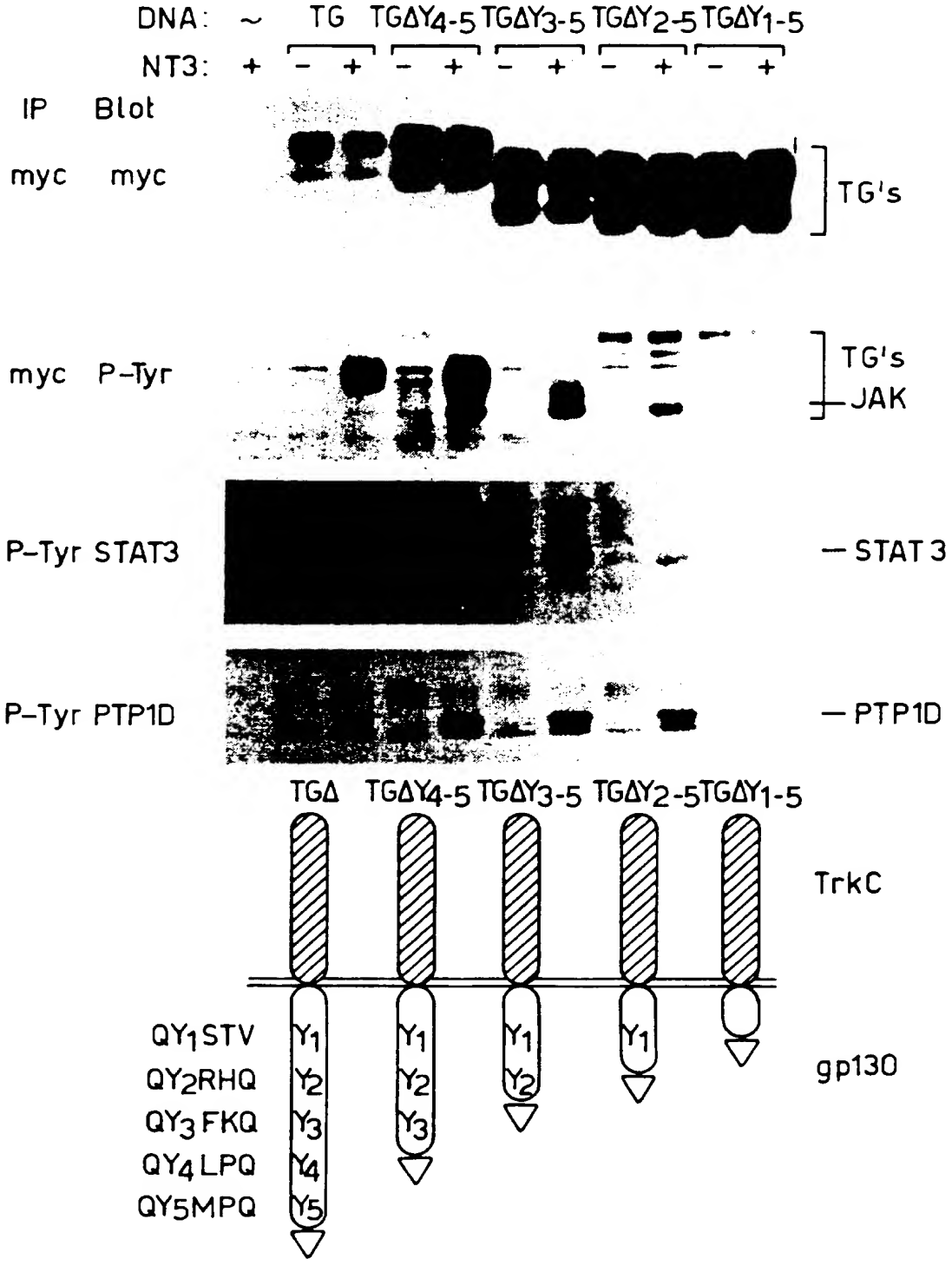
14. A nucleic acid according to claim 6 substantially as hereinbefore described with reference to any one of the foregoing Examples.

15. An expression vector according to claim 7 substantially as hereinbefore described with reference to any one of the foregoing Examples.

16. A cell according to claim 8 substantially as hereinbefore described with reference to any one of the foregoing Examples.

17. A method according to claim 9 substantially as hereinbefore described with reference to any one of the foregoing Examples.

Fig.1A.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TG's
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STAT3
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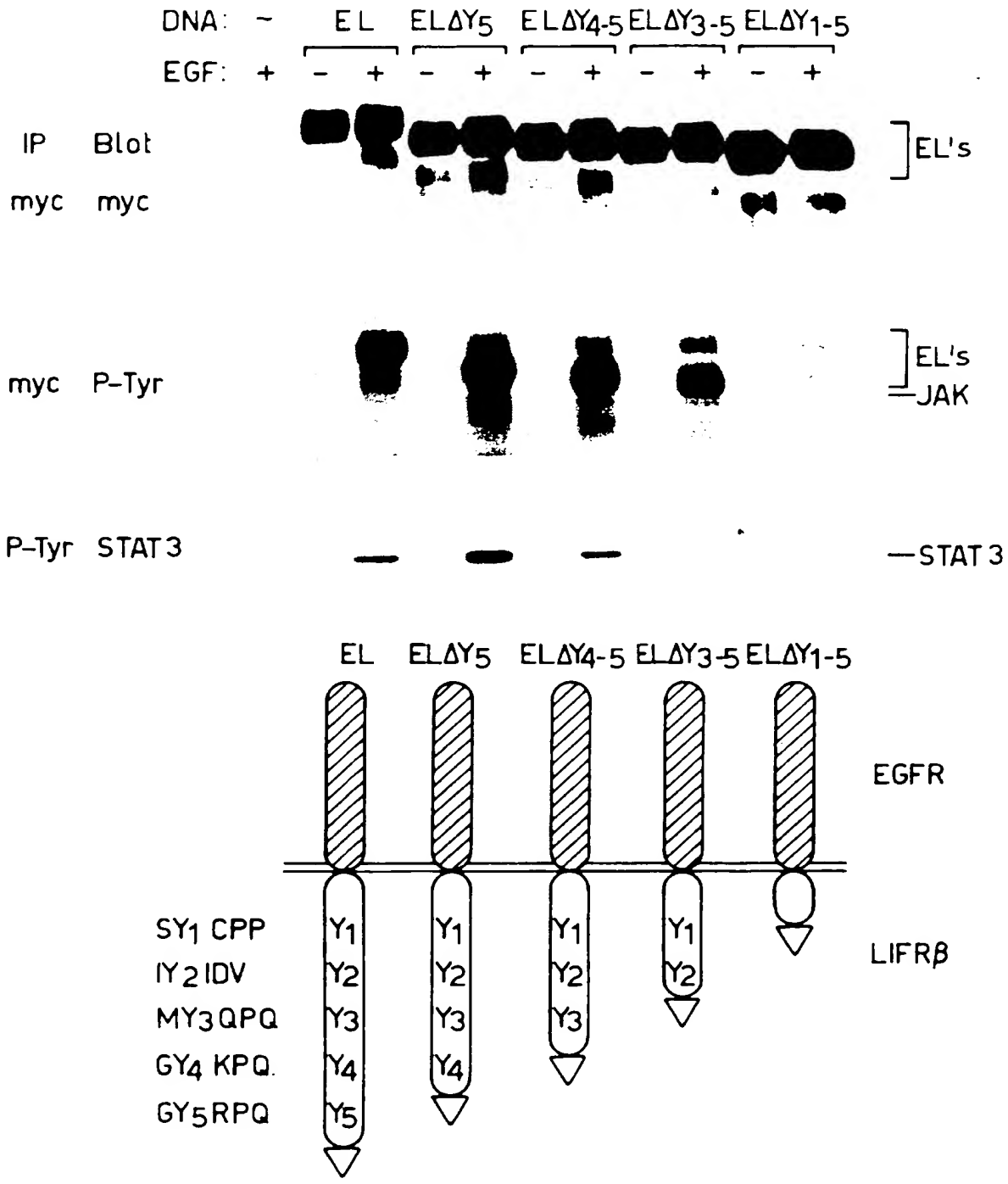
Fig.1B.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EL's
JAK
P-Tyr STAT3
STAT3
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EGFR
SY1 CPP
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GY5RPQ
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Y2
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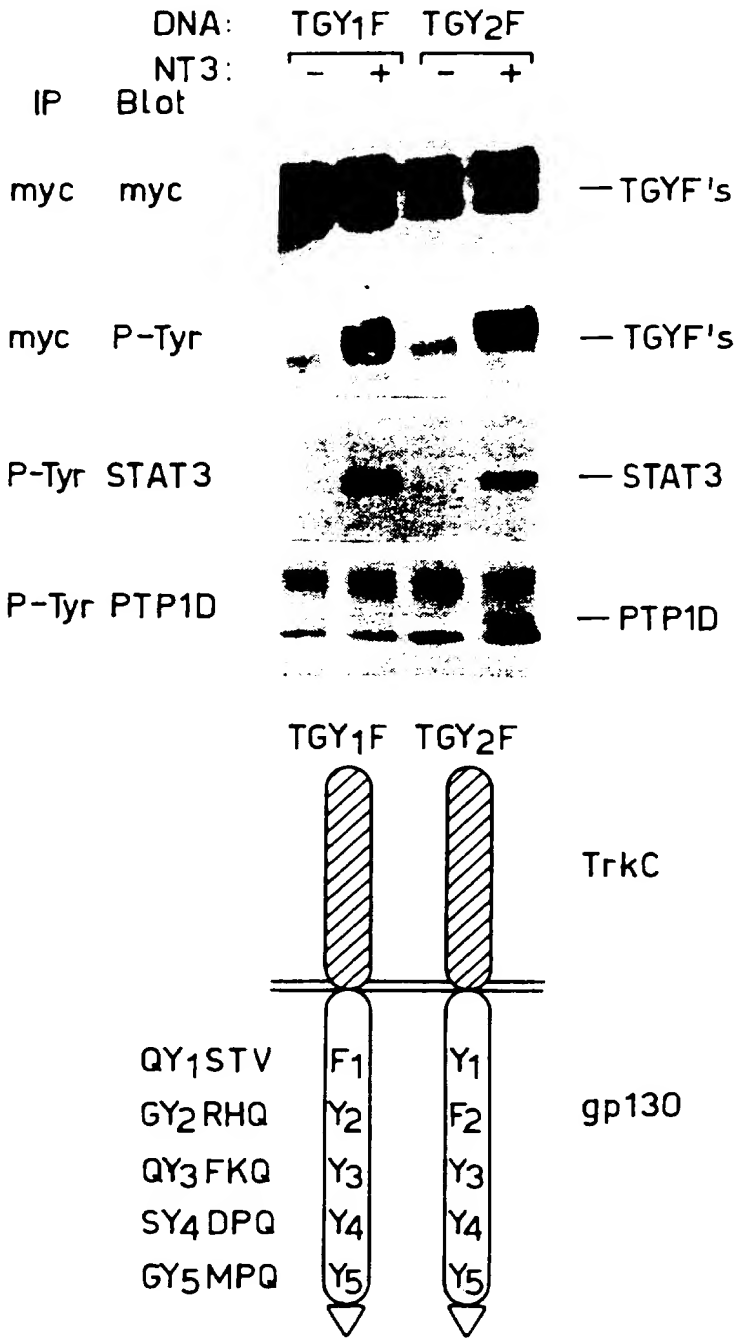
Y4
Y5
LIFRβ

Fig.1C.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— STAT3
P-Tyr PTP1D
— PTP1D
TGY1F TGY2F
TrkC
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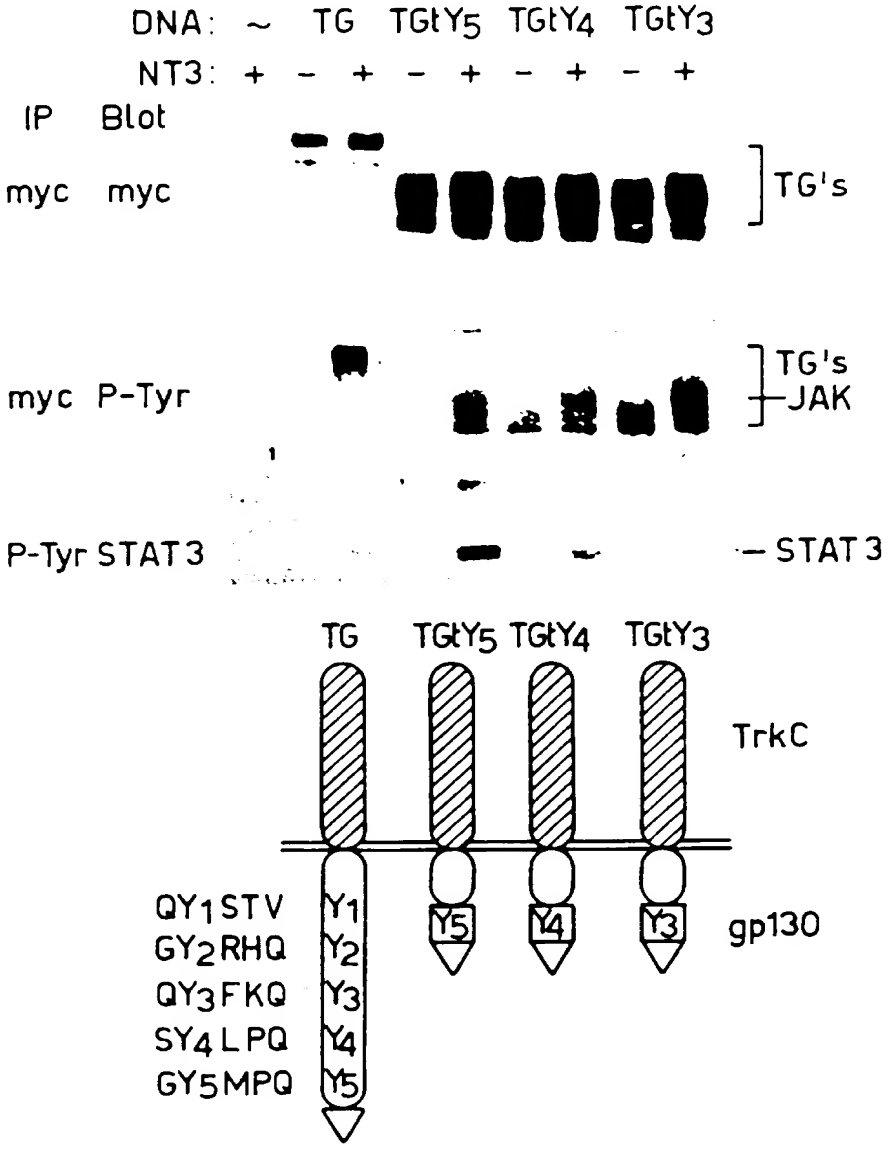
Fig.2A.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STAT3
TG
TGtY5
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TGtY3
TrkC
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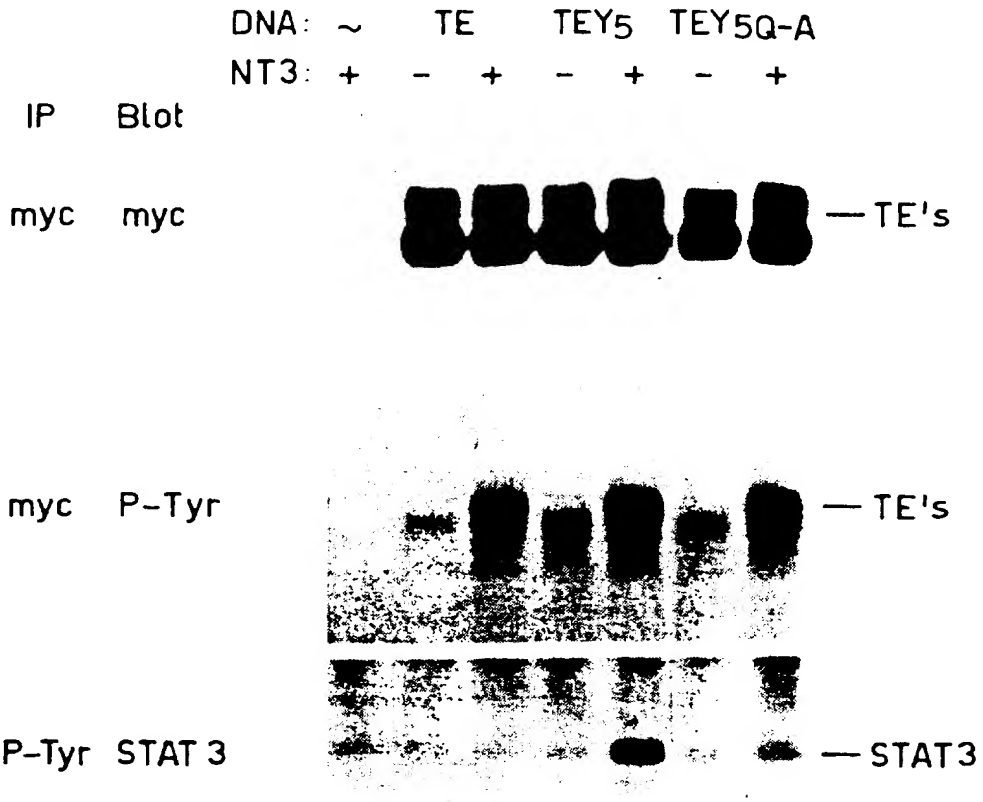
gp130

Fig.2B.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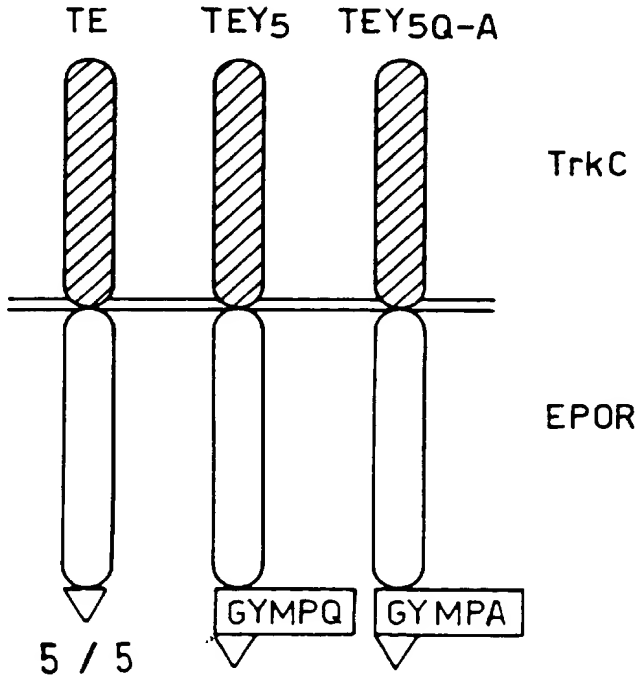
— STAT3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TrkC
EPOR
GYMPQ
GYMPA

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/09952

A. CLASSIFICATION OF SUBJECT MATT[redacted]
IPC 6 C12N15/62 C12N[redacted]5 C07K14/715 C07K14/705 [redacted]12N5/10
A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 1, 4 January 1994 WASHINGTON US, pages 158-162, HIDEYA OHASHI ET AL. 'Ligand-induced activation of chimeric receptors between the erythropoieitin receptor and receptor tyrosine kinases' cited in the application see abstract see page 158, left column, paragraph 1 - paragraph 2 see page 158, right column, paragraph 2 see page 159, right column, paragraph 2 - page 160, left column, paragraph 1 see page 161, right column, paragraph 2 - page 162, left column, paragraph 4 --- -/--	1,6-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 December 1995	03.01.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Montero Lopez, B

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INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 95/09952

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 10151 (IMMUNEX CORPORATION) 27 May 1993 cited in the application see page 3, line 15 - line 25 see page 6, line 13 - line 29 see page 7, line 27 - page 8, line 5 see page 13, line 34 - page 14, line 7 see page 24, line 3 - line 15	1,6-12

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible]nal Application No
PCT/US 95/09952

Patent document cited in search report	[illegible]blication date	Patent family member(s)		Publication date
WO-A-9310151	27-05-93	US-A-	5262522	16-11-93
		AU-A-	3228593	15-06-93
		CA-A-	2124085	27-05-93
		EP-A-	0672061	20-09-95
		JP-T-	7501941	02-03-95
		US-A-	5426048	20-06-95